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L3	L2 and (histochemical or tnbt or magnesium (w) chloride or sodium (w) azide or nicotiamide(w) diphosphate or nadp or dihydrofolic (w) acid)	154	L3
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- ☐ 61. 6143716. 07 Oct 98; 07 Nov 00. Liposomal peptide-lipid conjugates and delivery using same. Meers; Paul R., et al. 514/2; 424/450 514/16 514/17 514/18 530/324 530/326 530/327 530/328 530/386 530/402. A61K038/00 A61K009/127 C07K005/00 C07K007/00.
- ☐ 62. 6120994. 23 May 97; 19 Sep 00. Antioxidant responsive element. Tam; Shui-Pang. 435/6; 435/320.1 435/325 435/375 435/455 536/24.1. C12Q001/68 C07H021/04.
- ☐ 63. 6118047. 19 Jan 96; 12 Sep 00. Anthranilate synthase gene and method of use thereof for conferring tryptophan overproduction. Anderson; Paul C., et al. 800/278; 435/108 435/183 435/252.3 435/320.1 435/419 536/23.2 800/298 800/320.1. C12N005/04 C12N015/82 C12N015/29 A01H005/00.
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- ☐ 67. 6087094. 26 Feb 98; 11 Jul 00. Compositions and methods for detecting viral infection. Scholl; David R., et al. 435/5; 435/325 435/366. C12Q001/70 C12N005/10.
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L3: Entry 80 of 154

File: USPT

Dec 7, 1999

DOCUMENT-IDENTIFIER: US 5998159 A

TITLE: Methods for screening for antibioticsAbstract Text (1):

The present invention provides methods for identification of antibiotic agents which cause the accumulation of ppGpp in bacterial organisms involving a relA-independent pathway. The methods comprise screening assays in which test compounds are brought into contact with relA.sup.- test cells and observing the effect such compounds have on ppGpp levels in the test cells. The invention also provides genetically manipulated relA.sup.- test cells which contain a reporter gene the expression of which is sensitive to the level of ppGpp. The invention also encompasses agents identified by the screening assays, and uses of these agents in the treatment of infectious diseases.

Brief Summary Text (2):

The present invention relates to methods for high-throughput screening for compounds with antibiotic activity. Specifically, the invention relates to high-throughput screens which target mechanisms for accumulation of the nucleotide guanosine-3',5'-bis-pyrophosphate ("ppGpp") by relA.sup.+ independent pathways in bacteria. Such targets include ppGpp synthetase II (PSII) and ppGpp degradase (SpoT), both encoded by the spoT locus in bacteria. The invention further relates to novel compounds identified using such screening methods.

Brief Summary Text (4):

Resistance to currently available antibiotics has created a need for new antibiotic agents. In the United States alone, 19,000 hospital patients die each year due to nosocomial (hospital-acquired) bacterial infections (Service, R., 1995, Science 270:724-727). These infections, caused by organisms such as Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecium and Enterococcus faecalis, have become increasingly resistant to currently approved antibiotics. For example, significant clinical problems include methicillin-resistant strains of S. aureus, which are resistant to all current antibiotics except vancomycin (a drug of last resort because of severe side effects), and a vancomycin-resistant strain of E. faecium enterococci which is now found world-wide. The occurrence of vancomycin-resistant enterococci isolated from nosocomial infections rose from 0.4% to 13.6% in the relatively short time span from 1989 to 1993 (Tenover, F. C. and Hughes, J. M., 1996, JAMA 275(4):300-304) (reporting statistics from the Centers for Disease Control and Prevention). Even community-acquired organisms such as Streptococcus pneumoniae are increasingly resistant to antimicrobial agents, with a significant number of isolates being resistant to penicillin and extended-spectrum cephalosporins. Id.

Brief Summary Text (5):

The emergence and spread of resistant bacterial organisms are primarily caused by acquisition of drug resistance genes, resulting in a broad spectrum of antibiotic resistance (e.g., extended-spectrum cephalosporin-resistant mutant .beta.-lactamases found in several bacterial organisms). Genetic exchange of multiple-resistance genes, by transformation, transduction and conjugation; combined with selective pressures in settings such as hospitals where there is heavy use of antibiotic therapies, enhance the survival and proliferation of antimicrobial agent-resistant bacterial strains occurring by, e.g., spontaneous mutants. Id. Although the extent to which bacteria develop resistance to antimicrobial drugs and the speed with which they do so vary with different types of drugs, resistance has inevitably developed to all antimicrobial agents (Gold and Moellering, Jr., 1996, New Eng. J. Med., 335(19):1445-1453).

Brief Summary Text (8):

Because resistance to antibiotics is assuming even greater clinical importance, there is a pressing need to develop more effective methods for antibiotic drug discovery. Traditional approaches to screening for antimicrobial agents include chemical modification of existing drugs and mass screening of compounds for bacterial growth inhibition. The first approach, chemical modification of existing antibiotics, attempts to circumvent bacterial resistance while finding more potent activities. This approach has shown some success, however, it does not produce new classes of drugs and is unlikely to identify new bacterial processes as targets for drug intervention. The second is to directly test compounds for their ability to inhibit bacterial growth using standard microbiological methods, such as growth inhibition assays where libraries of natural products, semisynthetic, or synthetic chemicals are screened for their ability to kill or arrest growth of the target pathogen or a related nonpathogenic model organism. These tests are useful in that they are fast, uncomplicated, relatively inexpensive and allow for rapid testing of large libraries of compounds. However, such screens are blind to the compound's mechanism of action so that rate of efficacy, selectivity, and resistance remain elusive. That is, the promising lead compounds that emerge from such screens must not only be tested for possible toxicity to the human, animal, or plant host, they also undergo detailed mechanism-of-action studies conducted to identify the affected molecular target and precisely how the drug interacts with this target.

Brief Summary Text (9):

Another approach involves screening for compounds which target the resistance mechanism of known antibiotics. The compounds are then administered in conjunction with known antimicrobial agents. This technique is currently being tested for treating organisms resistant to tetracycline compounds. Tetracycline resistant organisms do not accumulate tetracycline within the cell and actually excrete the drug by cellular efflux pumps. Compounds which are tetracycline analogs that tightly bind to the pumps are given in conjunction with tetracycline to assist tetracycline in reaching its target in the bacterial cell (Service, 1995, Science 270:724-727), supra. This approach, however, is complicated and does not affect the underlying resistance mechanism.

Brief Summary Text (10):2.2. DECIPHERING MECHANISMS OF ANTIBIOTIC ACTIONBrief Summary Text (11):

Once antibiotics are identified, a number of studies can be performed to determine their mechanism of action and their selectivity. Such analyses can sometimes provide new understanding of basic cellular mechanisms.

Brief Summary Text (12):

For example, sulfonamides (or sulfa drugs), the first important antimicrobial agents identified, are actually antimetabolites and not antibiotics. Sulfanilamide, one of the sulfonamide class drugs, is a structural analog of para-aminobenzoic acid ("PABA"). The mode of action of sulfanilamide was unknown until it was discovered that PABA is required for the synthesis of the essential vitamin, folic acid. Folic acid synthesis is required for bacterial growth since bacteria are not capable of folic acid uptake. Sulfonamides inhibit the bacterial synthesis of folic acid by acting as competitive inhibitors of PABA. For humans, folic acid is also an essential vitamin, but unlike bacteria, humans are capable of uptake of folic acid and can obtain the vitamin through diet. As a result, bacteria, but not humans, are vulnerable to sulfa drugs which inhibit folic acid synthesis. In the sulfa class alone, thousands of chemically modified derivatives have been studied with about 25 of them still in use.

Brief Summary Text (13):

Similarly, much has been learned about peptidoglycan synthesis since the discovery of the penicillin and cephalosporins (peptidoglycan is the critical component in maintaining the shape and rigidity of both Gram positive and Gram negative bacterial organisms). Therefore the discovery of new classes of drugs can broaden the general understanding of bacterial physiology as well as provide for new antibacterial chemotherapeutics.

Brief Summary Text (14):2.3. THE USE OF ANTIBIOTICS TO STUDY THE METABOLISM OF ppGppBrief Summary Text (15):

Guanosine-3',5'-bis-pyrophosphate or guanosine tetraphosphate (ppGpp) is a nucleotide which inhibits bacterial growth when it accumulates intracellularly. In the enteric bacterium *Escherichia coli*, there are two enzymes which catalyze the synthesis of ppGpp. One of the enzymes is ppGpp synthetase I (PSI) and is encoded by the *relA* locus. The PSI enzyme is activated during amino acid starvation which results in what is known as the stringent response (Cashel, 1969, J. Biol. Chem. 244:3133-3141; Cashel and Gallant, 1969, Nature 221:838-841). Although the stringent response was first characterized as a response to amino acid starvation, it is now recognized that ppGpp levels change in response to a variety of stress conditions, including carbon, nitrogen or phosphate starvation, heat shock, osmotic shock and pH changes (Murray and Bremer, 1996, J. Mol. Biol. 259:41-57; Cashel et al., 1996, *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, ed. Neidhardt, F.C. ASM Press, Washington, D.C. pp. 1458-1496). The second ppGpp synthetic enzyme is ppGpp synthetase II (PSII), and is encoded by the *spoT* Locus.

Brief Summary Text (16):

Certain compounds, including several antibiotics, have been used as tools to study the metabolism of ppGpp in bacteria. For example, Cortay & Cozzzone (Cortay & Cozzzone, 1983, *Biochimica et Biophysica Acta*, 755:467-473) used polymyxin B and gramicidin, antibiotics which disrupt the cell membrane and thereby exert an antibacterial effect, to study ppGpp since a secondary effect of these antibiotics is a decrease in the rate of ppGpp degradation. The assays used by Cortay and Cozzzone to study the metabolism of ppGpp involved measuring the intracellular levels of nucleotides following drug treatment or amino-acid starvation of bacteria. As a general screening method, such an approach would be time consuming and better suited to studying the metabolic effect of individual agents.

Brief Summary Text (17):

The effects of various antibiotics which interfere with protein synthesis were also investigated for effects on ppGpp degradation. Tetracycline, chlortetracycline and thiostrepton were shown to strongly inhibit ppGpp degradation in vitro, and levallorphan (a morphine analogue) moderately inhibited ppGpp degradation in vitro (Richter, 1980, *Arch. Microbiol.* 124:225-332).

Brief Summary Text (18):

However, many of these results obtained in vitro have little bearing in vivo where direct contact of the degradase with the unmetabolized antibiotic may not occur. In the case of tetracycline, the inhibition was ascribed to chelation of manganese at high concentrations of the antibiotic. The concentrations required would not be achievable in vivo.

Brief Summary Text (19):

An antibiotic produced by *Xenorhabdus luminescens*, 3,5-dihydroxy-4-ethyl-trans-stilbene ("ES"), is thought to inhibit bacterial cell growth via an increase in ppGpp concentration by the *relA*.sup.+ -dependent mechanism (Sundar & Chang, 1992, *Antimicrobial Agents and Chemotherapy* 36(12):2645-2651). Sundar & Chang reported that ES inhibited growth of a stringent *E. coli* (*relA*+), but did not inhibit growth of an isogenic relaxed strain (*relA*-). In addition, ES caused ppGpp to accumulate in a *relA*+ strain but not in *relA*--strain. Furthermore, ES did not appreciably interfere with ppGpp degradase. Together these properties strongly suggested that ES action is to induce *relA*-dependent ppGpp synthesis, which leads to inhibition of growth.

Brief Summary Text (21):

The invention relates to high-throughput screens which target ppGpp accumulation in bacterial organisms by *relA*.sup.+ (PSI) independent pathways. Such targets include, for example and not by way of limitation, ppGpp synthetase II (PSII) and 3'-pyrophosphohydrolase, ("ppGpp degradase") both of which are encoded by the *spoT* locus. The ppGpp degradase enzyme is also referred to as the *spoT* enzyme, "ppGpp hydrolase", and "ppGppase" in the literature.

Brief Summary Text (22):

The invention is based, in part, on applicants' recognition that compounds which cause ppGpp accumulation in a *relA*.sup.+ (PSI) independent manner, such as by enhancing PSII activity or inhibiting ppGpp degradase, have the potential to be therapeutically beneficial as an antibiotic. This antibiotic property exists since increasing levels of ppGpp inhibit many cellular processes, including ribosomal RNA synthesis, and thus, ribosomes, which are essential for bacterial survival and growth. Deletion of the *spoT* gene, removes both PSII and ppGpp degradase and is not tolerated in the presence of a wildtype *relA* allele (Sarubbi et al., 1988, Mol. Gen. Genet., 213:214-222), suggesting that accumulation of ppGpp is lethal. Apparently, an adequate level of degradase in the organism is essential to prevent accumulation of toxic levels of ppGpp. Compounds which, for example, reduce, or inhibit this level of degradase activity are attractive candidates for therapeutic use. Similarly, compounds which enhance PSII activity are also desired. Accordingly, the invention provides methods for screening for potential antibiotic drugs by isolating compounds that, for example, enhance PSII ppGpp production or inhibit ppGpp degradase at the *spoT* locus in bacteria.

Brief Summary Text (25):

Bacterial strains without PSI activity (e.g., *relA* mutated strains) are advantageously used in the screens of the invention since this eliminates the potential for isolating compounds which causes ppGpp synthesis by the PSI enzyme and allows for the identification of compounds that specifically affect PSI-independent factors in ppGpp accumulation. This is significant since bacteria which lose the PSI gene by mutation are not uncommon and occur readily among laboratory isolates of *E. coli*. Elimination of PSI dependent synthesis, using *relA* mutant strains, allows for specific targeting of ppGpp degradase and/or PSII synthase.

Brief Summary Text (33):

In another embodiment, the invention relates to novel compounds identified by using such screening methods, as well as the uses of these compounds for developing antibiotic drugs for treatment of infectious diseases in animals.

Brief Summary Text (35):

The methods of the present invention provide an efficient, focused approach to drug discovery with significant improvements over previous methods. One major improvement is a method for increasing the efficiency of drug discovery by ensuring that lead compounds are more likely to affect their desired molecular target inside the test organism. Unlike the traditional approaches used for antimicrobial drug screening, which are either blind to the bacterial mechanism which is being targeted and/or do not provide for new classes of antibiotics, the present invention targets a unique bacterial process. The screening methods described herein can identify compounds that cause ppGpp accumulation by *relA* independent mechanisms, such as, enhanced PSII activity or inhibition of ppGpp degradase. Using the present approach new classes of compounds may be found, and efficacy and selectivity of these compounds can be optimized.

Brief Summary Text (56):

Test compounds are obtained from a wide variety of sources including collections of natural products in the form of bacterial, fungal, plant and animal extracts; and synthetical chemical libraries. Numerous means known in the art are available for the random, directed and combinatorial synthesis of a wide variety of chemical structures. In addition, natural products or known antibiotic compounds may be subjected to random or directed chemical modifications to produce derivatives and structural analogs for use as test compounds in the invention. Usually various predetermined concentrations are used for screening such as 0.001 .mu.M, 0.01 .mu.M, 0.1 .mu.M, 1.0 .mu.M, 10 .mu.M, and 100 .mu.M.

Brief Summary Text (62):

Bacteria generally exist in rapidly changing nutritional environments. To optimize growth and division in these various environments, bacteria developed sophisticated gene control mechanisms to allow the organism to produce particular enzymes in quantities that is optimal for the environment. Similarly, the translation apparatus itself is controlled by growth conditions.

Brief Summary Text (63):

For purposes of the treatment of human diseases caused by bacteria, understanding these gene control mechanisms provides an opportunity to look for compounds which adversely affect the bacterial genes that control growth. With this in mind, applicants have developed a strategy for screening for potential antibiotic drugs by identifying compounds which cause an accumulation of ppGpp via a relA independent pathway such as, for example, enhancing PSII activity or inhibiting ppGpp degradase. The assays of the invention provide a highly sensitive system that can be used to detect compounds that would otherwise be overlooked by ppGpp assays which have been used to study gene function in bacteria.

Brief Summary Text (65):

ppGpp appears to be responsible for negative regulation of rRNA synthesis during the stringent response. In *E. coli*, the PSI enzyme encoded at the relA is activated during amino acid starvation. The PSI enzyme is located on the ribosome and is activated by codon-specific binding of uncharged tRNA in the ribosomal RNA acceptor site (Haseltine and Block, 1973, Proc. Natl. Acad. Sci. USA 70:1564-1568; Cochran and Byrne, 1974, J. Biol. Chem. 249:353-360; Pedersen and Kjeldgaard, 1977, Eur. J. Biochem 76:91-97).

Brief Summary Text (71):

PSII (ppGpp synthetase II), encoded by the SpoT gene, is the second known enzyme which catalyzes the synthesis of ppGpp. PSII is one of the drug target in the present invention. PSII is not activated by amino acid starvation but is active during exponential growth and during certain conditions of environmental stress e.g., phosphate source starvation (Hernandez and Bremer, 1993, J. Biol. Chem. 268:10851-10862). Levels of ppGpp during exponential growth are determined by PSII activity. Id.

Brief Summary Text (72):

Relatively less is known about the products of the SpoT gene. The *E. coli* PSII enzyme has not been characterized biochemically and attempts to obtain homogenous protein from *E. coli* have been unsuccessful suggesting that the enzyme is functionally or physically unstable (Richter, 1969, In Ribosomes (Chambliss, Gaven, Davis, Davis, Kahan and Nomura eds.) 743-765, Univ. Park Press, Baltimore, Md.). PSII activity has, however, been found in certain *Bacillus* organisms (Sy and Akers, 1976, Biochemistry 15:4399-4403; Fehr and Richter, 1981, J. Bacteriol. 145:68-73). PSII activity is found in the cytoplasm. *ibid.* SpoT (ppGpp degradase) is the major enzyme activity causing turnover of ppGpp.

Brief Summary Text (73):

It had been suggested that the SpoT gene encodes a single product which may be a bifunctional enzyme capable of catalyzing both ppGpp synthesis and degradation, or that the SpoT gene could encode a regulation of both degradation or synthesis of ppGpp (Cashel and Rudd, 1987; Metzger et al., 1989, J. Biol. Chem. 264:9122-9125; Hernandez and Bremer, 1991, J. Biol. Chem. 266(9):5991-5999; Xiao et al., 1991, J. Biol. Chem. 266(9):5980-5990). PSII activity is reported to be generated during or shortly after spoT mRNA translation. Regulation of spoT encoded activities appears to be responsible for the growth medium-dependent changes in basal levels of ppGpp. PSII appears to catalyze ppGpp synthesis in a ribosome independent fashion. Although the identity of the PSII enzyme has not been proven, the evidence that spoT is the structural gene for PSII and is not simply asserting a regulatory effect is based on the fact that ppGpp is not detectable in strains with deletions in both relA and spoT (Xiao et al., 1991, J. Biol. Chem. 266(9):5980-5990) and the fact that RelA and SpoT have extensive amino acid sequence homology throughout their length. Metzger et al., 1989 J. Biol. Chem. 264:9122-9125.

Brief Summary Text (74):

Further, analysis of the *E. coli* spoT gene has recently identified distinct but overlapping regions involved in ppGpp synthesis and degradation (Gentry and Cashel, 1996, Molecular Microbiol. 19(6):1373-1384). The region containing the first 203 amino acids of the 702 amino acid SpoT protein was confirmed to have ppGpp degradase activity. An overlapping region containing residues 67-374 conferred PSII activity. Id. In addition, PSII activity of *B. stearothermophilus* appears to reside in a monomeric enzyme whose molecular mass is about the same as that of the *E. coli* SpoT

product. Fehr and Richter, 1981 J. Bacteriol. 145:68-73.

Brief Summary Text (88):

In one specific embodiment, the expression of the reporter gene in test cells is placed under the control of a promoter that is negatively regulated by ppGpp. By negative regulation is meant that the promoter is transcriptionally active in the absence of ppGpp, and is transcriptionally inactive in the presence of ppGpp. That is, the reporter gene product is produced only when ppGpp levels are low. Under normal conditions, the test cells produce some ppGpp by the PSII enzyme, but this ppGpp is degraded by the degradase activity, and the overall ppGpp levels are low. When the ppGpp level is low, the reporter gene is expressed. In the presence of a compound which, for example, enhances PSII activity or inhibits ppGpp degradase, ppGpp accumulates and as a result, transcription of the reporter gene is repressed. The lack of expression or downregulation of expression of the reporter gene indicates a rise in ppGpp level in the test cells, and identifies the test compound as a candidate for further studies.

Brief Summary Text (91):

The preferred assay strains accumulate a low level of ppGpp from an equilibrium of the PSII enzyme and ppGpp degradase activities. Under these conditions, the lacZ reporter gene is expressed and the assay strain is effectively lac.sup.+. In the presence of a compound which, for example, enhances PSII activity or causes ppGpp degradase inhibition, ppGpp accumulates to high levels and therefore transcription of lacZ is inhibited, and the assay strain becomes effectively lac.sup.-.

Brief Summary Text (98):

The test cells are plated on rich medium on which the test cells grow and produce some ppGpp by the PSII enzyme. But this ppGpp is degraded by the degradase activity and the overall ppGpp levels are low. Under these conditions, there is only a low background level of reporter gene expression from the positively regulated promoter, since ppGpp is required for full expression. In the presence of a compound which causes the accumulation of ppGpp to high levels, such as, for example, a ppGpp degradase inhibitor, ppGpp accumulates and therefore the reporter gene is fully expressed.

Brief Summary Text (103):

It should be noted that, since in vitro and in vivo conditions provide different environments, certain compounds which give a certain result in vitro result will not necessarily give the same result in vivo. For example, two compounds that have been reported to inhibit ppGpp degradase in vitro did not test positive in the assay of the invention: tetracycline and picolinic acid. Both have been reported to indirectly inhibit by chelation of manganese (manganese is required for ppGpp degradase activity).

Brief Summary Text (104):

In the case of tetracycline, inhibition was reported in an in vitro reaction when the antibiotic was added to a final concentration of about 400 .mu.g/ml (Richter, 1980, Arch. Microbiol. 124:229-232). This concentration would be impossible to achieve in whole cells since concentrations above 50 .mu.g/ml completely inhibit bacterial growth. Thus, the lack of a red ring is explained by the discrepancy between the concentration needed for degradase inhibition and the concentration that can be tolerated by growing bacteria.

Brief Summary Text (114):

Escherichia coli can be used as a model of many bacteria. Test compounds that specifically inhibit host transcription of E. coli test cells are expected to have a similar inhibitory effect on the transcription of pathogenic species, such as but not limited to, Vibrio species, Pseudomonas species, Acinetobacter species, Bordetella species, Campylobacter species, Haemophilus species, Neisseria species and Enterobacteriaceae species, such as Salmonella, Enterobacter, Klebsiella, Yersinia, Proteus, Serratia, and Staphylococcus species, Streptococcus species, Corynebacterium species, Listeria species and Bacillus species. It is also expected that positive test compounds will be effective as an antibiotic against multidrug-resistant strains of these pathogenic species, such as .beta.-lactam-resistant strains of E. coli.

Brief Summary Text (121):

The host cells which contain the reporter gene sequence and which express the reporter gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions (e.g., resistance to antibiotics); (c) assessing the level of transcription as measured by the expression of reporter mRNA transcripts in the host cell; and (d) detection of the reporter gene product as measured by immunoassay or by its biological activity.

Brief Summary Text (127):

For convenience and efficiency, enzymatic reporters and light-emitting reporters are preferred for the screening assays of the invention. Accordingly, the invention encompasses histochemical, colorimetric and fluorometric assays.

Brief Summary Text (128):

A variety of enzymes may be used as a reporter which includes but are not limited to .beta.-galactosidase (Nolan et al. 1988, Proc. Natl. Acad. Sci. USA 85:2603-2607), chloramphenicol acetyltransferase (CAT; Gorman et al., 1982, Mol Cell Biol, 2:1044; Prost et al., 1986, Gene 45:107-111), .beta.-lactamase, .beta.-glucuronidase and alkaline phosphatase (Berger et al., 1988, Gene 66:1-10; Cullen et al., 1992, Methods Enzymol; 216:362-368). Transcription of the reporter gene leads to production of the enzyme in test cells. The amount of enzyme present can be measured via its enzymatic action on a substrate resulting in the formation of a detectable reaction product. The methods of the invention provides means for determining the amount of reaction product, wherein the amount of reaction product generated or the remaining amount of substrate is related to the amount of enzyme activity. For some enzymes, such as .beta.-galactosidase, .beta.-glucuronidase and .beta.-lactamase, fluorogenic substrates are available that allow the enzyme to covert such substrates into detectable fluorescent products (see, for example, U.S. Pat. No. 5,070,012, and WO 96/30540).

Brief Summary Text (129):

The most preferred reporter gene of the invention is the LacZ gene encoding E. coli .beta.-galactosidase. The enzyme is very stable and has a broad specificity so as to allow the use of different chromogenic or fluorogenic substrates, such as but not limited to lactose 2,3,5-triphenyl-2H-tetrazolium (lactose-tetrazolium), 5-bromo-4-chloro-3-indolyl-.beta.-D-galactoside (X-gal), and fluorescein galactopyranoside (Molecular Probes, Orgeon). See, Nolan et al. 1988, Proc. Natl. Acad. Sci. USA 85:2603-2607.

Brief Summary Text (130):

Another commonly used reporter gene is the E. Coli .beta.-glucuronidase gene (GUS; Gallagher, 1992, in "GUS protocols", Academic Press) which can be used with various histochemical and fluorogenic substrates, such as X-glucuronide, and 4-methylumbelliferyl glucuronide.

Brief Summary Text (131):

A variety of bioluminescent, chemiluminescent and fluorescent proteins can also be used as light-emitting reporters in the invention. One type of such reporters, which are enzymes and require cofactor(s) to emit light, include but are not limited to, the bacterial luciferase (luxAB gene product) of Vibrio harveyi (Karp, 1989, Biochim. Biophys. Acta 1007:84-90; Stewart et al. 1992, J Gen Microbiol, 138:1289-1300), and the luciferase from firefly, Photinus pyralis (De Wet et al. 1987, Mol. Cell. Biol. 7:725-737).

Brief Summary Text (133):

Depending on the screening technique and nature of the signal used to assay the reporter gene expression, a reporter regimen can be used to aid directly or indirectly the generation of a detectable signal by a reporter molecule. A reporter regimen comprises compositions that enable and support signal generation by the reporter, such as substrates and cofactors for reporter molecules that are enzymes; e.g., lactose-tetrazolium medium. Such compositions are well known in the art. Components of a reporter regimen may be supplied to the test cells during any step of the screening assay.

Detailed Description Text (17):

In this embodiment, the assay strain is a *relA* deficient mutant with reduced PSI synthetase activity but with normal PSII synthetase activity and normal degradase activity with a *lacZ* reporter gene under the control of a promoter which is positively controlled by ppGpp. This strain is plated as a lawn on solid medium, and test compounds are added in wells, as described in Section 5.4.1. The medium can be LB (as described in Maniatis et al., MOLECULAR CLONING A Laboratory Manual, Cold Spring Harbor Press, eds. 1982) or similar rich medium containing X-gal (80 .mu.g/ml) as a chromogenic substrate for the .beta.-galactosidase enzyme. Colony color around the well or disk containing test compound is compared to control areas of the plate that are free of test compound. Comparison of test and control areas is done at the same time point. Compounds that cause ppGpp accumulation are expected to show a ring of blue growth around the well or disk. In other lactose differential medium, results will be analogous but the positive and negative readout will depend on the indicator of lactose fermentation. A known inhibitor of ppGpp degradase, such as NaN.sub.3, 1,10-phenanthroline or picolinic acid is included in every plate as a positive control. At high concentrations this compound is toxic, and therefore there is a zone of inhibited bacterial growth close to the disk. At some distance from the disk, a ring of blue growth is seen, indicating that at some lower concentration, NaN.sub.3 is allowing ppGpp accumulation and the cells have become lac.sup.+.

Detailed Description Text (32):

Active ppGpp degradase (SpoT protein) can be isolated from *E. coli* and used in an in vitro enzyme assay in order to assess the effects of compounds on the partially purified enzyme.

Detailed Description Text (34):

Ribosomes with active ppGpp synthetase I (*relA*) are prepared from a strain of *E. coli* that carries a plasmid encoding the *relA* gene under the control of an inducible promoter. These ribosomes are then combined with GTP and ATP, and the *relA* protein synthesizes both ppGpp and pppGpp. CF3120 cells are grown in Luria broth containing 100 .mu.g/ml ampicillin to an A.sub.600 of 1.5. *relA* expression is then induced by the addition of IPTG to a final concentration of 1 mM. Cells are incubated for 1 hour, then harvested by centrifugation. The cell pellet is washed in ribosomal buffer (50 mM Tris acetate [pH 8.0], 15 mM Mg acetate, 60 mM potassium acetate, 27 mM ammonium acetate, 1 mM DTT and 0.2 mM EDTA) and the resulting cell pellet is stored at -70.degree. C. The frozen cell pellet is resuspended in 2 volumes (w/v) of ribosomal buffer then cells are lysed by French press. The lysate is centrifuged at 11,000.times.g for 40 min at 4.degree. C. The supernatant is centrifuged at 30,000 rpm in a Beckman Ti65 (or equivalent) for 4 hrs at 4.degree. C. The resulting pellet of ribosomes and membranes is combined with 2.5 volumes of cold ribosomal buffer, transferred to a beaker and stirred slowly overnight at 4.degree. C. The solution is then centrifuged at 7,500.times.g for 15 min at 4.degree. C. to remove undissolved debris. The supernatant is removed and ribosomal buffer is added to bring the suspension to 4.times. (w/v) with respect to the original weight of the cells. A 5 ml cushion of 40% sucrose in ribosomal buffer is placed in a 30 ml ultracentrifuge tube then the ribosomal suspension is carefully layered on top, filling the tube. The preparation is centrifuged at 32,000 rpm in a Beckman Ti65 (or equivalent) for 4 hrs at 4.degree. C. The supernatant is discarded and the pellet is transferred to a beaker containing a minimal volume of cold ribosomal buffer. The mixture is stirred at 4.degree. C. until resuspended then stored by dropping drops into a beaker filled with liquid nitrogen. The drops freeze and can be stored in vials at -70.degree. C. Just before use, drops are transferred to a tube and thawed on ice.

Detailed Description Text (38):

Active ppGpp degradase is prepared from a strain of *E. coli* that overexpresses spoT. In a preferred embodiment, overexpression of spoT is accomplished by induction of an IPTG-responsive promoter in a plasmid carrying the gene for spoT. Cells are grown at 30.degree. C. in Luria broth supplemented with 0.2% glucose, 40 mM potassium phosphate (pH 7.5) and appropriate antibiotics. When the culture reaches an A600 of 1.0, expression of spoT is induced by the addition of IPTG to a final concentration of 1 mM. Cells are incubated for an additional 2 hrs, then harvested by centrifugation, washed in lysis buffer (50 mM Tris acetate, [pH 8.0], 5 mM EDTA, 0.23 M NaCl, 100 .mu./ml PMSF, 1 mM DTT), then stored at -80.degree. C. The frozen

cell pellet is resuspended in 3 volumes of lysis buffer and cells are lysed using a French press. The resulting cell lysate is centrifuged at 11,000.times.g. SpoT protein is found in both the supernatant and the pellet. SpoT protein is extracted from the pellet by adding 50 ml TGED buffer containing 1M NaCl and stirring at 4.degree. C. for 1 hr (TGED buffer=10 mM Tris, [pH 8.0], 5% glycerol, 0.1 mM EDTA, 0.1, 0.1 mM DTT). Ammonium sulfate is added to the extracted pellet solution and to the supernatant, to a final volume of 25%. The mixture is stirred at 4.degree. C. for 1 hr, then precipitated proteins (including SpoT) are recovered by centrifugation at 11,000.times.g. The pellet is dissolved in 7 ml of TGED buffer containing 1M NaCl and 50% glycerol, then applied to a 200 ml gel filtration column (for example, Sephacryl 200) equilibrated with TGED containing 1M NaCl. Fractions containing SpoT protein are pooled and dialyzed against SpoT storage buffer (TGED buffer containing 1M NaCl and 10% glycerol). The resulting SpoT preparation is stored at -70.degree. C. The preparation contains partially purified active SpoT protein and can be used for enzyme assays. If further purification is desired, the preparation is applied to a 5 ml heparin column (for example, BioRad) that has been equilibrated with 300 mM NaCl, 5% glycerol. SpoT protein is eluted with a 100 ml continuous gradient of 300 mM to 1M NaCl, 5% glycerol. Fractions containing SpoT are pooled and stored at -70.degree. C. in SpoT storage buffer.

Detailed Description Text (46):

5.10. ANTIBIOTIC AGENTS IDENTIFIED BY METHODS OF THE INVENTION

Detailed Description Text (47):

In yet another embodiment, the invention provides novel antibiotic agents discovered by the methods described above. These antibiotic agents are capable of causing ppGpp accumulation in a bacterial cell, leading to downregulation of rRNA synthesis, and ultimately to a reduction or inhibition of bacterial growth. These agents may, for example, act by enhancing PSII activity, and/or inhibiting ppGpp degradase activity, and are expected to be effective in a variety of species of bacteria, including infectious pathogenic bacteria. The invention also includes novel pharmaceutical compositions which comprise antibiotic agents discovered as described above formulated in pharmaceutically acceptable formulations.

Detailed Description Text (48):

In another embodiment, the invention features a method for treating a subject infected with an infectious agent by administering to that subject a therapeutically effective amount of an antibiotic agent which causes ppGpp accumulation (for example, by enhancing PSII activity, and/or inhibiting ppGpp degradase activity) in the infectious agent as determined by the assays of the invention. Such administration can be by any method known to those skilled in the art, for example, by topical application or by systemic administration.

Detailed Description Text (49):

In yet another embodiment, antibiotic agents of the present invention can be used to treat contaminated items, such as crops, wood, metal or plastic and the like, by methods such as, but not limited to, spraying or dusting of that agent onto the contaminated item, or impregnating that agent into the item.

Detailed Description Text (52):

The antibiotic compounds identified by methods of the invention may be formulated into pharmaceutical preparations for administration to animals for treatment of a variety of infectious diseases. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may be prepared, packaged, labelled for treatment of and used for the treatment of the indicated infectious diseases caused by microorganisms, such as those listed infra in Section 5.9.3.

Detailed Description Text (53):

If the antibiotic compound is water-soluble, then it may be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, polyethylene glycol or glycerine. Thus, the compounds and their physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal,

parenteral, topical, dermal; vaginal, rectal administration and drug delivery device, e.g., porous or viscous material, such as lipof foam.

Detailed Description Text (59):

The antibiotic compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

Detailed Description Text (60):

In addition to the formulations described previously, the antibiotic compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the antibiotic compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

Detailed Description Text (61):

The antibiotic compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Detailed Description Text (62):

The pharmaceutical compositions of the present invention comprise an antibiotic compound as the active ingredient, or a pharmaceutically acceptable salt thereof, and may also contain a pharmaceutically acceptable carrier, and optionally, other therapeutic ingredients, for example antivirals. The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic acids and bases, including inorganic and organic acids and bases.

Detailed Description Text (64):

In practical use, an antibiotic agent can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including tablets, capsules, powders, intravenous injections or infusions). In preparing the compositions for oral dosage form any of the usual pharmaceutical media may be employed, e.g., water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, and the like; in the case of oral liquid preparations, e.g., suspensions, solutions, elixirs, liposomes and aerosols; starches, sugars, micro-crystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like in the case of oral solid preparations e.g., powders, capsules, and tablets. In preparing the compositions for parenteral dosage form, such as intravenous injection or infusion, similar pharmaceutical media may be employed, e.g., water, glycols, oils, buffers, sugar, preservatives and the like known to those skilled in the art. Examples of such parenteral compositions include, but are not limited to Dextrose 5% w/v, normal saline or other solutions.

Detailed Description Text (66):

For administration to subjects, antibiotic compounds discovered by using the assays of the invention are formulated in pharmaceutically acceptable compositions. The compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These compositions can be utilized in vivo, ordinarily in a mammal, preferably in a human, or in vitro. In employing them in vivo, the compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonially, rectally, vaginally, nasally, orally, transdermally, topically, ocularly, or intraperitoneally.

Detailed Description Text (67):

As will be readily apparent to one skilled in the art, the magnitude of a

therapeutic dose of an antibiotic compound in the acute or chronic management of an infectious disease will vary with the severity of the condition to be treated, the particular composition employed, and the route of administration. The dose, and perhaps dose frequency, will also vary according to the species of the animal, the age, body weight, condition and response of the individual subject. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art.

Detailed Description Text (68):

Desirable blood levels may be maintained by a continuous infusion of an antibiotic compound as ascertained by plasma levels. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

Detailed Description Text (71):

Antibiotic agents may also be systemically administered. Systemic absorption refers to the accumulation of agents in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: oral, intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ocular. Each of these administration routes exposes the agent to an accessible target.

Detailed Description Text (73):

The antibiotic compounds identified by the methods of the invention can be used to treat infectious diseases in animals, including humans, companion animals (e.g., dogs and cats), livestock animals (e.g., sheep, cattle, goats, pigs, and horses), laboratory animals (e.g., mice, rats, and rabbits), and captive or wild animals.

Detailed Description Text (74):

Specifically, infectious diseases caused by bacteria including but not limited to, gram positive cocci, such as Staphylococci (e.g., *S. aureus*), Streptococci (e.g., *S. pneumoniae*, *S. pyogenes*, *S. faecalis*, *S. viridans*); gram positive bacilli, such as Bacillus (e.g., *B. anthracis*), Corynebacterium (e.g., *C. diphtheriae*), Listeria (e.g., *L. monocytogenes*); gram negative cocci, such as Neisseria (e.g., *N. gonorrhoeae*, *N. meningitidis*); gram negative bacilli, such as Haemophilus (e.g., *H. influenzae*), Pasteurella (e.g., *P. multocida*), Proteus (e.g., *P. mirabilis*), Salmonella (e.g., *S. typhi*, *S. murium*), Shigella species, Escherichia (e.g., *E. coli*), Klebsiella (e.g., *K. pneumoniae*), Serratia (e.g., *S. marcescens*), Yersinia (e.g., *Y. pestis*), Providencia species, Enterobacter species, Bacteroides (e.g., *B. fragilis*), Acinetobacter species, Campylobacter (e.g., *C. jejuni*), Pseudomonas (e.g., *P. aeruginosa*), Bordetella (e.g., *B. pertussis*), Brucella species, Francisella (e.g., *F. tularensis*), Clostridia (e.g., *C. perfringens*), Helicobacter (e.g., *H. pylori*), Vibrio (e.g., *V. cholerae*), Mycoplasma (e.g., *M. pneumoniae*), Legionella (e.g., *L. pneumophila*), Spirochetes (e.g., *Treponema*, *Leptospira* and *Borrelia*), Mycobacteria (e.g., *M. tuberculosis*), Nocardia (e.g., *N. asteroides*), Chlamydia (e.g., *C. trachomatis*), and Rickettsia species, can be treated by antibiotic drugs discovered by the methods of the invention.

Detailed Description Text (82):

16 ml of the 1:5000 dilution was pipetted and added directly to the surface of a 10.times.10 inch plate containing lactose-tetrazolium medium (as described in Silhavey et al., Experiments with Gene Fusions, Cold Spring Harbor Press, 1984, ppGpp. 268-9). The lactose-tetrazolium plate contained approximately 300 ml of medium in a square plaque tray (Stratagene, Inc.). The medium contained 25.5 g BBL-Base, antibiotic medium 2 (Becton Dickinson Microbiology Systems) 50 mg 2,3,5-triphenyl-2H tetrazolium chloride and 50 ml 20% beta-lactose solution per liter. The plate was swirled slowly to cover the entire surface evenly with the diluted culture. After the culture covered the agar plate surface, the plate was tipped diagonally so that the remaining liquid accumulated in one corner. Using a sterile pipet, the remaining liquid was removed from the plate. The plate was then placed, lid off, in a laminar flow hood to dry for 2 hours.

Detailed Description Paragraph Table (1):

positive controls: phenanthroline, 10 mg/ml
polymyxin E, 10 mg/ml negative controls: spectinomycin, 10 mg/ml tetracycline, 1
mg/ml

Detailed Description Paragraph Table (2):

positive controls: phenanthroline, 10 mg/ml
polymyxin E, 10 mg/ml negative controls: spectinomycin, 10 mg/ml tetracycline, 1
mg/ml

Other Reference Publication (19):

Heinemeyer & Richter, 1977, "In vitro degradation of guanosine tetraphosphate (ppGpp) by the enzyme associated with the ribosomal fraction from Escherichia coli", FEBS Lett. 84:357-361.

Other Reference Publication (47):

Sundar & Chang, 1992, "The role of guanosine-3',5'-bis-pyrophosphate in mediating antimicrobial activity of the antibiotic 3,5-dihydroxy-4-ethyl-trans-stilbene", Antimicrob. Agents Chemother. 36:2645-2651.



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TITLE: Ryegrass pollen allergen

Detailed Description Text (7):

Accordingly, it is within the scope of the present invention to encompass all proteins belonging to the Lol p Ib family, at least one fragment (peptide) of a Lol p Ib protein family member, and amino acid derivatives thereof, and to encompass nucleotide sequences, including DNA, cDNA and mRNA and homologue or degenerate forms thereof, encoding Lol p Ib family members or fragments thereof, or derivatives thereof. It is also within the scope of the invention to encompass purified native Lol p Ib, at least one fragment (peptide) thereof, and derivatives or homologues thereof. It is further in accordance with the present invention to include molecules such as polypeptides fused to a Lol p Ib protein, or at least one fragment thereof, or derivatives thereof or to nucleotide sequences contiguous to such fragment and/or derivative-encoding nucleotide sequences. For example, for some aspects of the present invention, it is desirable to produce a fusion protein comprising a Lol p Ib family member or at least one fragment thereof or their derivatives and an amino acid sequence from another peptide or protein, examples of the latter being enzymes such as beta-galactosidase, phosphatase, urease and the like. Most fusion proteins are formed by the expression of a recombinant gene in which two coding sequences have been joined together such that their reading frames are in phase.

Alternatively, proteins or peptides can be linked in vitro by chemical means. All such fusion protein or hybrid genetic derivatives of a Lol p Ib protein or its encoding nucleotide sequences are encompassed by the present invention. Furthermore, by homologues and derivatives of a Lol p Ib protein is meant to include synthetic derivatives thereof. The nucleotide sequences as elucidated herein, can be used to chemically synthesize the entire protein or generate any number of fragments (peptides) by chemical synthesis by well known methods (eg solid phase synthesis). All such chemically synthesized peptides are encompassed by the present invention. Accordingly, the present invention extends to isolated Lol p Ib protein family members, fragments thereof and their derivatives, homologues and immunological relatives made by recombinant means or by chemical synthesis.

Detailed Description Text (16):

For expression in *E. coli*, suitable expression vectors include pTRC (Amann et al. (1988) Gene 69: 301-315); pET-11d (Novagen, Madison, Wis.); PGEX (Amrad Corp., Melbourne, Australia); pMAL (N.E. Biolabs, Beverly, Mass.); pRIT5 (Pharmacia, Piscataway, N.J.); and pSEM (Knapp et al. (1990) BioTechniques 8: 280-281). The use of pTRC and pET-11d will lead to the expression of unfused protein. The use of pGEX, pMAL, pRIT5 and pSEM will lead to the expression of allergen fused to glutathione S-transferase (PGEX), maltose E binding protein (pMAL), protein A (pRIT5), or truncated beta-galactosidase (pSEM). When a Lol p Ib protein family member, fragment, or fragments thereof is expressed as a fusion protein, it is particularly advantageous to introduce an enzymatic cleavage site at the fusion junction between the carrier protein and the Lol p Ib protein family member or fragment thereof. A Lol p Ib family member or fragment thereof may then be recovered from the fusion protein through enzymatic cleavage at the enzymatic site and biochemical purification using conventional techniques for purification of proteins and peptides. Suitable enzymatic cleavage sites include those for blood clotting Factor Xa or thrombin for which the appropriate enzymes and protocols for cleavage are commercially available from for example Sigma Chemical Company, St. Louis, Mo. and N.E. Biolabs, Beverly, Mass.

Detailed Description Text (35):

The allergenic nature of the subject proteins are characterized in part, by their

binding of the reaginic IgE antibodies which are present at high levels in sera of allergic patients. The IgE binding to the epitopes on allergic proteins can be tested in a chromogenic assay in which allergens immobilized on a solid support can be visualized by sequential incubation in (1) allergic patients serum; (2) enzyme-labelled anti-IgE antibodies.

Detailed Description Text (43):

Well established methods exist for introducing recombinant DNA molecules into plant cells such as use of Agrobacterium plasmids and electroporation amongst others. By "deleterious function" in respect of a polypeptide refers to a feature of said polypeptide that will inhibit cell growth, cause lysis of a cell, or inhibit various functions in a cell and thereby prevent the normal functioning of the cell. In this case, lethal gene constructs having a deleterious function are contemplated which inhibit or prevent pollen formation and thereby result in a male sterile plant. Such "lethal genes" may encode enzymes, enzyme inhibitors, and/or toxic polypeptides, amongst other molecules. Alternatively, the lethal gene may encode an antisense RNA capable of inhibiting translation of a particular species of mRNA, the translated product thereof, being vital for pollen development.

Detailed Description Text (46):

Accordingly, the present invention provides a recombinant DNA molecule comprising a ryegrass pollen promoter sequence, and in particular a promoter for a gene encoding a Lol p Ib family member, or homologues or degenerate forms thereof located on said molecule and further having one or more restriction endonuclease sites downstream of said promoter such that a nucleotide sequence inserted into one or more of these sites is transcribable in the correct reading frame and is thereby a developmentally regulated, pollen-specific expression vector. As used herein, the "correct reading frame" has the same meaning as "in phase". The aforementioned DNA molecule will preferably also have a selectable marker thereon, such as an antibiotic or other drug resistance gene, such as for example gene encoding resistance to ampicillin, carbenicillin, tetracycline, streptomycin and the like. The recombinant molecule will further comprise a means for stable inheritance in a prokaryotic and/or eukaryotic cell. This can be accomplished by said recombinant molecule carrying a eukaryotic and/or a prokaryotic origin of replication as hereinbefore described in relation to expression vectors.

Detailed Description Text (63):

By "reporter molecule," as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e., radioisotopes). In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chose for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for peroxidase conjugates, 1,2-phenylenediamine, 5-aminosalicylic acid, or toluidine are commonly used. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the tertiary complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells or latex beads, and the like.

Detailed Description Text (66):

Accordingly, the present invention is also directed to a kit for the rapid and convenient assay for antibodies to Lol p Ib or derivatives, homologues or immunological relatives thereof in mammalian body fluids (e.g., serum, tissue extracts, tissue fluids), in vitro cell culture supernatants, and cell lysates. The kit is compartmentalized to receive a first container adapted to an antigenic component thereof, and a second container adapted to contain an antibody to Lol p Ib, said antibody being labelled with a reporter molecule capable of giving a detectable signal as hereinbefore described. If the reporter molecule is an enzyme, then a third container adapted to contain a substrate for said enzyme is provided. In an exemplified use of the subject kit, a sample to be tested is contacted with the contents of the first container for a time and under conditions for an antibody, if present in the sample, to bind to Lol p Ib protein in said first container. If the Lol p Ib protein of the first container has bound to antibodies in the test fluid, the antibodies of the second container will bind to the secondary complex to form a tertiary complex and, since these antibodies are labelled with a reporter molecule, when subjected to a detecting means, the tertiary complex is detected. Therefore, one aspect of the present invention is a kit for the detection of antibodies to a protein having allergenic properties, said protein from pollen of the family Poaceae (Gramineae), the kit being compartmentalized to receive a first container adapted to contain recombinant Lol p Ib protein or its antigenic derivative or homologue or a purified native Lol p Ib protein or its antigenic derivative or homologue, and a second container adapted to contain an antibody to Lol p Ib or derivative or homologue thereof, said antibody labelled with a reporter molecule capable of giving a detectable signal. The "reporter molecule" may also involve agglutination of red blood cells (RBC) on latex beads. In this kit the reporter molecule is a radioisotope, an enzyme, an fluorescent molecule, a chemilluminiscent molecule, bioluminescent molecule or RBC. The kit alternatively comprises a container adapted to contain recombinant Lol p Ib or is antigenic derivative or homologue labelled with a reporter molecule capable of giving a detectable signal.

Detailed Description Text (76):

The present invention, therefore, provides a pharmaceutical compositions comprising a desensitizing or therapeutically effective amount of Lol p Ib or derivatives, homologues or immunological relatives thereof and one or more pharmaceutically acceptable carriers and/or diluents. The active ingredients of a pharmaceutical composition comprising Lol p Ib is contemplated to exhibit excellent therapeutic activity, for example, in the desensitization of humans allergic to grass pollen when administered in amount which depends on the particular case. For example, from about 0.5 ug to about 20 mg per kilogram of body weight per day may be administered. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (e.g., using slow release molecules). Depending on the route of administration, the active ingredients which comprise the pharmaceutical composition of the invention may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredients. For example, Lol p Ib may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound, such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes. For purposes of inducing T cell anergy, the pharmaceutical composition if preferably administered in non-immunogenic form (e.g. it does not contain adjuvant).

Detailed Description Text (113):

A search for consensus glycosylation sequences (Asn-X-Ser/Thr) in the deduced amino acid sequence detected no such sequences. The absence of an N-linked carbohydrate chain on the allergen was confirmed by the lack of deglycosylation following treatment with the enzymes N-glycanase and endo-F glycosidase. Chemical

deglycosylation followed by SDS-PAGE showed no decrease in molecular weight of the protein. The 31/33 kD components remained as a doublet, suggesting that the difference in molecular weight is not due to glycosylation. The deglycosylation treatments did not affect IgE binding to the 31/33 kD components. As compared to Lol p Ia which has 5% carbohydrate, no carbohydrate is present in Lol p Ib.

Detailed Description Text (169):

Immunological screening was done by plating the libraries and duplicate filters were produced. One of the filters was screened with the pooled allergic sera and the other was screened with MAbs PpV1 and PpV4 provided by F. Matthiesen (ALK, Horsholm, Denmark). The bound antibodies were detected using standard chromogenic methods.

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Terms	Documents
L2 and (histochemical or tnbt or magnesium (w) chloride or sodium (w) azide or nicotiamide(w) diphosphate or nadp or dihydrofolic (w) acid)	154

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L3: Entry 1 of 10

File: USPT

Oct 29, 2002

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TITLE: Comparative phenotype analysis

Brief Summary Text (6):

Methods and identification systems to characterize microorganisms widely used in industry for production of food and drink (e.g., beer, wine, cheese, yogurt, etc.), the is production of antibiotics (e.g., penicillin, streptomycin, etc.), bioremediation of oil spills, biological control of insect pests (e.g., *Bacillus thuringiensis*), and the production of recombinant proteins, are still needed. In addition, very few identification methods and systems have been developed for environmental use and there remains a need for simple and generally useful identification methods of many organisms. In particular, methods for identification and growth of the actinomycetes are lacking.

Brief Summary Text (25):

Ever since Waksman isolated actinomycin in 1940, and streptomycin in 1943, the streptomycetes have attracted a large amount of attention (see e.g., G. S. Kobayashi, et al., at p. 671). Thousands of soil samples collected world-wide have resulted in the identification of over 90% of the therapeutically useful antibiotics (see e.g., G. S. Kobayashi, "Actinomycetes: The Fungus-Like Bacteria, in B. D. Davis et al. (eds.), Microbiology, 4th ed., J. B. Lippincott Co., Philadelphia [1990], pages 665-671). The interest in improving antibiotic qualities and yields has resulted in various studies on this group of organisms, including improved methods for their growth and characterization.

Brief Summary Text (26):

It is important that strains be differentiated in screening programs to identify antibiotic activities, so that redundant testing is avoided. In addition, differentiation facilitates determination of taxonomic relationships which may lead to other organisms with promising activities. Unfortunately, testing of these organisms is often very difficult. Because they grow as filaments, they have a strong tendency to form clumps of mycelia which makes them much more difficult to handle, both in liquid cultures and on solid or semi-solid agar media. Furthermore, because of their complex life cycle which involves sporulation and germination, it is very difficult to obtain cultures which perform consistently in metabolic and biochemical testing programs. In addition, the presence of spores and the potential for their inhalation, represents a safety hazard to personnel responsible for the cultivation and characterization of these organisms, especially in settings where large-scale growth is necessary (e.g., antimicrobial production).

Brief Summary Text (47):

In one embodiment, the testing substrates are selected from the group consisting of carbon sources and antimicrobials. In yet another embodiment, the method further includes a calorimetric indicator, wherein the colorimetric indicator is selected from the group consisting of chromogenic substrates, oxidation-reduction indicators, and pH indicators.

Brief Summary Text (51):

It is also contemplated that the kit will also include a calorimetric indicator selected from the group consisting of chromogenic substrates, oxidation-reduction indicators, and pH indicators.

Brief Summary Text (55):

It is also contemplated that the kit will include a colorimetric indicator selected from the group consisting of chromogenic substrates, oxidation-reduction indicators,

type of the redox initiators, the transfer of electrons can be effected directly or with the aid of a so-called electron transfer agent. Examples of possible electron transfer agents include phenazine methosulfate, phenazine ethosulfate, meldola blue and diaphorase.

Brief Summary Text (26):

The bacteria to be tested for sensitivity towards antibiotics can be used, for example, as pure cultures which have been obtained from blood agar, casein peptone-soya meal peptone agar, brotacin agar or similar nutrient media. The bacterial suspension in the test batch should usually have a germ count of 10.sup.5 -10.sup.8 germs/ml.

Detailed Description Text (3):

The test solution consists of twice-concentrated Muller-Hinton broth in 0.1 M phosphate buffer, pH 7.0, which is 2.times.10.sup.-5 M in 4-methylumbelliferyl .beta.-D-galactopyranoside and 2.times.10.sup.-5 M in octylphenol polyethylene glycol ether and which contains the abovementioned ampicillin concentrations. After incubation of the test batch at 37.degree. C. for 30 minutes, the microtiter plate is irradiated with a UV lamp at 366 nm. Inhibition of murein biosynthesis by ampicillin is to be recognized in fluorescence of the test batch as a result of significant .beta.-D-galactosidase activity in comparison with the control without the antibiotic or batches with antibiotic concentrations below the minimum inhibitory concentration. The test batches containing 4.0 and 8.0 .mu.g/ml of ampicillin exhibit significant fluorescence, that is to say murein biosynthesis of *Enterobacter cloacae* is inhibited under the chosen test conditions at these ampicillin concentrations.

Detailed Description Text (11):

Inhibition of murein biosynthesis by ampicillin is to be recognized in a significantly lower extinction of the test batch at 340 nm in comparison with the control without the antibiotic or batches with antibiotic concentrations below the minimum inhibitory concentration. The test batches containing 8.0 and 16.0 .mu.g/ml of ampicillin exhibit a significantly lower extinction at 340 nm, that is to say a significant malate dehydrogenase activity can be detected in these test batches. Under the chosen test conditions, murein biosynthesis in *Citrobacter freundii* is inhibited at 8.0 and 16.0 .mu.g/ml of ampicillin.

Detailed Description Text (13):

A kit for testing the sensitivity of a bacterium towards an antibiotic which acts primarily by disrupting murein biosynthesis comprises a deep drawn component with 6 cavities of about 100 .mu.l in size. The cavities contain in lyophilized form an antibiotic free control and 5 different antibiotic concentrations in a buffered nutrient medium together with the fluorogenic or chromogenic enzyme substrate and a detergent.

Current US Original Classification (1):

435/32

CLAIMS:

1. A method for testing the sensitivity of a bacterium toward an antibiotic which acts primarily by disrupting ^{*β -lactam*} murein biosynthesis in growing bacteria, comprising,

culturing the bacterium in a nutrient medium therefor which also contains an amount of the antibiotic equal to or greater than its minimum inhibitory concentration for the bacterium, and a substrate for a cytoplasmic enzyme of the bacterium, wherein said medium is substantially not effective to burst cells of the bacterium having a normal murein component, but is effective to burst bacterial cells having an abnormal murein component, and

determining the presence of a substantial amount of the cytoplasmic enzyme by observing a property of the resultant medium which is sensitive to the interaction of the enzyme with its substrate,

whereby the presence of a substantial amount of the cytoplasmic enzyme indicates the

sensitivity of the bacterium toward the antibiotic.

6. A method of claim 5 wherein the hydrolytic enzyme substrate is a fluorogenic or chromogenic enzyme substrate.

8. A method of claim 7 wherein the medium further comprises a coenzyme for the oxidoreductase or a fluorogenic or chromogenic redox indicator.

12. A method of claim 11 wherein, in the medium, the concentration of the antibiotic is up to 64 $\mu\text{g/ml}$, of the substrate is 10^{-1} to 10^{-5} M, of the bacteria is $10^{5.5}$ to $10^{8.8}$ germs/ml and of the detergent is 10^{-4} to 10^{-6} M.

13. A method of claim 1 wherein the antibiotic has a β -lactam structure.

14. A method claim 1 wherein the antibiotic is a penicillin or a cephalosporin.

15. A method of claim 1 wherein the culturing of the bacterium is further conducted separately in individual samples of the nutrient medium containing different concentrations of the antibiotic.

16. A method of claim 15 wherein the culturing is also simultaneously conducted in a nutrient medium containing no antibiotic or containing less than its minimum inhibitory concentration.

19. A kit for testing the sensitivity of a bacterium towards an antibiotic which acts primarily by disrupting murein biosynthesis, comprising separate containers, each containing a sample of buffered nutrient medium for the bacterium and each containing a different concentration of the antibiotic, a substrate for a cytoplasmic enzyme of the bacterium, and an amount of a detergent effective to burst cells of the bacterium which have an abnormal murein component but essentially ineffective to burst cells having a normal murein component, wherein at least one concentration of the antibiotic is equal to or greater than its minimum inhibitory concentration for the bacterium.

21. A kit of claim 19 wherein the antibiotic has a β -lactam structure.

22. A kit of claim 19 wherein the concentrations of the antibiotic are up to 64 g/ml, of the substrate are 10^{-1} to 10^{-5} M, and of the detergent are 10^{-4} to 10^{-6} M.